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A Microcapillary Column Switching HPLC−Electrospray Ionization MS System for the Direct Identification of Peptides Presented by Major Histocompatibility Complex Class I Molecules

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A microcapillary column switching high-performance liquid chromatography (HPLC) system was developed for the separation of major histocompatibility complex (MHC) class I associated peptides. Combination of the column switching system with electrospray ionization mass spectrometry (ESIMS) enabled the detection and identification of the peptides at low-femtomole levels. Sample volumes of 30−50 μL were injected and concentrated onto a short, 100-μm-i.d. precolumn. The precolumn was coupled to a 100-μm-i.d. reversed-phase analytical HPLC column via a six-port valve. Peptides were separated on the analytical column using an ESI-compatible mobile phase at a flow rate of 0.5 μL/min. Peptides were eluted directly into the ESI source of either a magnetic sector MS or an ion trap MS. Peptides associated with human leukocyte antigen A*0201 molecules were determined in immunoaffinity-purified extracts from either measles virus infected cells or uninfected cells by microcapillary column switching HPLC−ESIMS. The approach toward detection of virus-specific peptides we used was based on the comparison of ion chromatograms obtained from the LC−MS analysis of extracts from virally infected cells and their uninfected counterparts. In this way, the molecular mass of peptides unique to virus infected cells was obtained. The utility of the system is demonstrated by the identification of a candidate epitope. Microcapillary column switching HPLC was used along with ESI ion trap tandem MS to identify the naturally processed viral peptide KLWESPQEI. This peptide was found to derive from the measles virus nonstructural protein C. The approach described here provides a versatile and sensitive method for the direct identification of viral peptides associated with MHC class I molecules.

Viruses establish their infections inside the host's cells, where antibodies cannot reach them. After a cell is infected, a sequence of events called antigen processing takes place inside the cell which is aimed at giving a signal to the immune system that the cell has been infected. First, proteins derived from the virus are degraded within the cytoplasm by proteasomes to form short peptides. These peptides are transported into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) molecule. Inside the endoplasmic reticulum, two proteins, the major histocompatibility complex (MHC) class I heavy chain and the β_2-microglobulin (β_2m), join together. The heavy chain, β_2m, and a peptide associate into a trimolecular complex, the MHC class I molecule. Finally, the MHC molecule is routed to its final destination on the cell surface. Cytotoxic T lymphocytes (CTL) identify infected cells by looking for viral peptides (T-cell epitopes) presented by a class I MHC molecule.[1] In this way, T lymphocytes can recognize and kill infected cells selectively, sparing healthy cells. MHC molecules also present peptides from normal cellular proteins. Even on the surface of a virally infected cell, the number of MHC molecules presenting viral peptides is only a small fraction of the total.

To allow the peptide to fit into the binding cleft of a class I MHC molecule, the length is restricted to 8−10 amino acid residues. Amino acid residues at particular positions of the peptide fit into pockets in the MHC binding cleft.[2] These pockets may allow only one or a few closely related amino acids (anchor residues) to bind. The binding mechanism of MHC molecules implies that peptides associated with a particular class I form must have some structural features in common. Indeed, this is what has been found.[3,4]

Until recently, the identification of specific MHC-associated peptides that are recognized by individual T cells has generally been accomplished by using peptide binding motifs to screen known source proteins and then testing the derived peptides for

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their ability to sensitize appropriate target cells for recognition.[5] Through technological advances in microchemistry and analytical chemistry, it has now become possible to identify peptides without prior knowledge of the source protein, i.e., direct identification. An important advantage of direct identification is the ability to detect posttranslational modifications.[6] Direct identification of individual peptides that comprise specific CTL epitopes in a mixture of thousands of similar peptides has proved difficult. Edman degradation has been utilized to sequence complex mixtures of antigenic peptides.[3] Although this method is useful to determine anchor residues, it does not normally allow the sequencing of individual peptides. Sequences of class I MHC-associated peptides have been obtained after high-performance liquid chromatography (HPLC) and Edman degradation.[7] However, HPLC is unable to completely resolve complex peptide mixtures and, when combined with Edman degradation, still only provides the sequence of the dominant peptides.

Electrospray ionization mass spectrometry (ESIMS) has emerged as a powerful technique for the analysis of species that exist as ions in solution, such as peptides.[8] ESIMS is readily coupled to reversed-phase high-performance liquid chromatography. HPLC−ESIMS allows accurate determination of the molecular weight of peptides from mixtures. The sequencing of peptides can be accomplished by applying ESI in conjunction with tandem mass spectrometry (MS/MS). The identification of T-cell epitopes demands the ability to sequence individual peptides in low-femtomole amounts from mixtures of thousands of peptides.[9] Because of the concentration sensitive behavior of electrospray ionization, low-femtomole detection limits can only be achieved by applying microcapillary (50−100 μm i.d. columns) HPLC−ESIMS. Hunt et al. were the first to demonstrate the feasibility of microcapillary HPLC−ESIMS for the analysis of peptides bound to class I and class II MHC molecules.[9,10] Since then this method has been successfully applied to the identification of MHC-associated peptides involved in melanoma,[11,12] ultraviolet-induced regressor tumor,[13] graft versus host disease,[14] and HIV infection.[15] Microcapillary LC columns only provide increased sensitivity compared to conventional-sized columns when the sample mass injected onto the column remains the same. Typically a 100-μm i.d. column is operated at a flow rate of 0.5 μL/min. Obviously it is not practical to inject microliter sample volumes at a flow rate of 0.5 μL/min. One way to circumvent this problem is to concentrate the sample volume to <1 μL. When working with small amounts of material, sample handling is often the limiting factor in an experiment; in this respect, sample concentration to <1 μL should be avoided. Samples can be pneumatically injected onto the column by using a high-pressure vessel containing the sample vial.[16] However, because of pressure limitations, microliter sample volumes can only be injected in a reasonable time when relatively short columns or columns packed with large particles (≥10 μm) are used.

In this article, we report the development and features of a microcapillary column switching system coupled to ESIMS. The system enables the injection of a relatively large sample volume (30−50 μL) onto a short precolumn in a matter of minutes. After the sample is loaded, the separation is performed on a 100-μm i.d. analytical column. The system was tailored to the analysis of MHC class I associated peptides. The suitability of the microcapillary column switching HPLC−ESIMS system for the analysis of viral peptides bound to MHC class I molecules is demonstrated. Naturally processed peptides were eluted from human leukocyte antigen (HLA)-A*0201 molecules purified from measles virus infected cells and uninfected cells and analyzed by microcapillary HPLC−ESIMS. A comparison of the results obtained from the HPLC−ESIMS analyses of extracts of infected and uninfected cells revealed the molecular mass of peptides that arose as a result of virus infection. By utilizing microcapillary HPLC along with tandem mass spectrometry, we identified a peptide unique to measles virus infected cells. To our knowledge, this is the first report that describes the successful identification of a naturally processed viral peptide with this approach.

Due to ever mutating viruses, mankind will probably always have to deal with virus infections. The aim of this work is to obtain a better understanding of antigen processing at the molecular level. The benefits could take the form of a better and more rational design of vaccines.

EXPERIMENTAL SECTION

Peptides. Angiotensin III and oxytocin were purchased from Sigma (St. Louis, MO). Synthetic peptides were made by solid-phase FMOC chemistry, using an ABIMED AMS 422 simultaneous multiple peptide synthesizer. The identities of synthetic peptides were confirmed by mass spectrometry. Peptides were dissolved in deionized water at a concentration of ~100 pmol/μL, and these stock solutions were stored at −20 °C. Stock solutions were diluted with 0.1 M aqueous acetic acid to a final concentration of 3.3 fmol/μL to 5 pmol/μL.

Cell Lines and Viruses. Human Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines expressing HLA A*0201 were used throughout this study. Cell lines were grown in RPMI 1640 medium supplemented with 10 mM HEPES, 2 mM glutamine, 100 units/mL penicillin and 100 μg/mL streptomycin (complete

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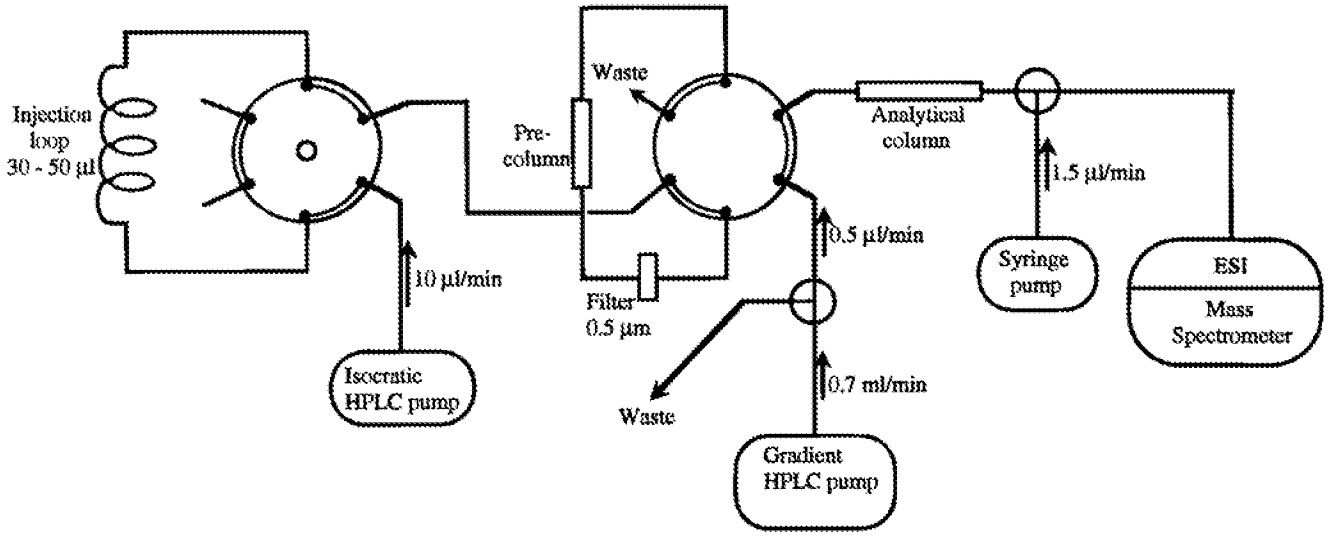

Figure 1. Schematic of the microcapillary column-switching HPLC–ESIMS system.

RPMI 1640 medium), and 10% heat-inactivated fetal calf serum (FCS) at 37 °C up to a total yield of (10–25) × 10^9 cells. Cells were harvested, washed four times with cold PBS, pelleted, and stored in batches of (3–5) × 10^9 cells at −70 °C. Virus-infected cell batches were prepared from EBV transformed cells after a 2-h infection with measles virus of the Edmonston B strain at a multiplicity of infection of 1:1 and subsequent incubation at 37 °C for 48–72 h in a complete RPMI 1640 medium supplemented with 2% FCS.

Immunoaffinity Purification and Extraction of MHC-Associated Peptides. HLA-A*0201 molecules were immunoprecipitated essentially as described previously.[9] Briefly, between 5 × 10^9 and 15 × 10^9 EBV transformed cells or their measles virus infected counterparts were lysed in a 50 mM Tris buffer (pH 8.0) containing 0.5% Nonidet P40 and protease inhibitors. After sample centrifugation at 10000*g* for 1 h, supernatants were passed over two CNBr-activated Sepharose 4B beads (Pharmacia Biotech, Uppsala, Sweden), the first coupled with normal mouse Ig and the second coupled with HLA-A*0201 specific monoclonal antibody. After several washing steps, peptides were eluted from immunoaffinity columns using 10% acetic acid (five bed volumes). The extracts were centrifuged at 3000*g* for 3.5 h through a 10-kDa macroseparation filter, concentrated to 500–1000 µL by vacuum centrifugation, and stored at −80 °C.

HPLC Fractionation of Peptide Extracts. Peptide extracts were fractionated by reversed-phase HPLC on a SMART (Pharmacia Biotech, Uppsala, Sweden) separation system. A 150-µL aliquot of the peptide extract was injected onto a Pharmacia C_2/C_{18} column (100 mm × 2.1 mm i.d., particle size 3 µm) and eluted with a 40-min linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 100 µL/min. Four fractions of 1–1.5 mL (designated 1–4) were collected, concentrated to ~10 µL by vacuum centrifugation, and stored at −70 °C. For HPLC–ESIMS analysis, 100 µL of 0.1 M acetic acid was added to the concentrated HPLC fractions.

Preparation of Microcapillary Columns. Microcapillary columns were prepared by a procedure similar to that described previously by Kennedy[17] and Moseley.[18]

Fused-silica capillary (Polymicro Technologies, Phoenix, AZ) with an inner diameter of 100 µm was cut to a length of ~50 cm. The outlet end of the capillary was tapped into a vial containing 5-µm spherical silica particles (LiChrosorb SI 60, Merck, Darmstadt, Germany) until a plug of ~1 mm was formed. A frit was constructed by sintering the particles into place by quickly passing the end of the capillary through the flame of a microtorch. A vial containing 2-propanol was placed into a stainless steel vessel connected to a cylinder containing helium. The inlet end of the fused-silica capillary was inserted through a $^1/_{16}$ in. inlet port in the lid of the vessel into the vial. The capillary was mounted into the fitting by using a vespel ferrule. The frit was tested by flushing 2-propanol through the capillary at a pressure of 70 bar. Subsequently, the frit was deactivated using a 5% solution of dimethyldichlorosilane in toluene (Supelco, Bellefonte, PA) and the capillary was flushed thoroughly with 2-propanol.

A slurry of 2-propanol and packing material was prepared in a vial. A Teflon-coated stir bar was placed in the vial, and the vial was put in the vessel. The vessel was mounted onto a magnetic stirrer, and the column was packed to the desired length by applying a pressure of ~70 bar.

Analytical columns were packed with Purospher RP-18 (Merck, Darmstadt, Germany) to a length of 20–25 cm. Precolumns were packed with 5-µm Alltima C_8 (Alltech, Deerfield, IL) to a length of 7–8 mm.

Microcapillary High-Performance Liquid Chromatography. The microcapillary column-switching HPLC–ESIMS system (Figure 1) consisted of a Gynkotek 300 (Germering, Germany) isocratic LC pump, a Perkin-Elmer (Norwalk, CT) 250 binary LC pump, a Rheodyne (Cotati, CA) 7725 injection valve, and a Valco (Houston, TX) EC6W six-port, two-position valve. All connections through which the sample passed were made from 50-µm i.d. fused-silica capillary.

Samples were injected onto the precolumn using an injection valve fitted with a 30-µL sample loop. The sample loop was made from 0.53-mm i.d. poly(ethylene glycol)-deactivated fused-silica tubing (SGE, Ringwood, Australia). Samples were loaded and

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preconcentrated on a 7 mm × 100 μm i.d. Alltima C_8 precolumn at a flow rate of 10 μL/min. The mobile phase used for preconcentration consisted of an aqueous solution of 0.1 M acetic acid. Six minutes after sample injection, the six-port valve was switched and the LC solvent gradient was started. The binary LC pump was operated at a flow rate of 700 μL/min. The solvent stream was split precolumn using a Valco tee and a piece of 50-μm-i.d. fused-silica capillary of sufficient length to ensure a flow rate through the analytical column of 0.5 μL/min. The sample was eluted from the precolumn and separated on a 250 mm × 100 μm i.d. Purospher RP-18 analytical column using a linear gradient of acetonitrile. The mobile phase used for gradient elution consisted of (A) 0.1 M acetic acid in water and (B) acetonitrile containing 0.1 M acetic acid. The gradient was linear from 0 to 10% B in 1 min, followed by 10 to 85% B in 25 min. A mixture of methanol and water (7:3 v/v) was added to the column effluent via a Valco microvolume tee. The water/methanol mixture was delivered at a flow rate of 1.5 μL/min by a Harvard Apparatus (South Natick, MA) model 22 syringe pump. The microvolume tee was connected to the electrospray probe by a piece of fused-silica capillary (50 μm i.d., 190 μm o.d.; Polymicro Technologies) which delivered the HPLC effluent to the tip of the electrospray needle.

Electrospray Ionization Mass Spectrometry. Mass spectra were obtained on a Finnigan MAT 95 mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an electrospray ionization source. An electrospray was generated by applying a voltage of 4.0 kV across the stainless steel electrospray needle and the heated capillary. The heated capillary was maintained at 250 °C. Positive ion mass spectra were acquired over various ranges of mass-to-charge ratio (m/z) at a resolution of ~1500 and a scan rate of 5 s/decade.

Tandem mass spectra were recorded on a Finnigan MAT LCQ (Finnigan MAT, San Jose, CA) ion trap mass spectrometer fitted with an electrospray ionization source. Electrospray ionization was performed by setting the electrospray voltage at 4.5 kV. The heated capillary temperature was held at 225 °C. Product ion spectra were generated by selecting either singly or doubly charged molecular ions ($[M + H]^+$ or $[M + 2H]^{2+}$) to undergo collision-induced dissociation (CID). The relative collision energy imparted to singly or doubly charged ions was 35 and 40%, respectively. The maximum injection time was 150 ms. The isolation width was set to 3.0 m/z units for singly charged parent ions and to 2.0 m/z units for doubly charged parent ions.

Derivatization with *N*-Succinimidyl-2-(3-pyridyl)acetate (SPA). On-column SPA derivatization was performed using a modification of a previously described procedure.[19] After a 30-μL aliquot of the sample was loaded onto a 7 mm × 100 μm i.d. Alltima C_8 precolumn, the sample loop was filled with a solution of SPA in a 0.1 M K_2HPO_4 buffer (pH 8.0). The SPA solution was prepared immediately before use by dissolving 1 mg of SPA in 1 mL of buffer solution. When the injection valve was turned to the inject position, the derivatization reagent was directed to the precolumn at a flow rate of 10 μL/min delivered by the isocratic HPLC pump (Figure 1). After 2 min, the injection valve was returned to the load position and the precolumn was washed free of interfering reagents with 0.1 M acetic acid for 6 min at a flow rate of 10 μL/min. Switching the six-port valve caused peptides to be eluted from the precolumn and separated on a 250 mm × 100 μm i.d. Purospher RP-18 analytical column using the previously described linear gradient of acetonitrile.

*N*Succinimidyl-2-(3-pyridyl)acetate was synthesized in our laboratory by the method of Sherman et al.[20]

RESULTS AND DISCUSSION

Selection of the Analytical Column. The most widely used mobile phase system for peptide separations using silica-based reversed-phase stationary phases is the aqueous acetonitrile system containing the weak hydrophobic ion-pairing reagent, trifluoroacetic acid (TFA). The low pH (pH 2) of the mobile phase suppresses the ionization of the residual silanol groups present at the surface of the silica, and hence, undesirable ionic interactions of the stationary phase with positively charged peptide residues are minimized. In addition, anions such as TFA form hydrophobic ion pairs with the positively charged amino acid residues of the peptides. The resulting complex tends to mask the positive charge and tends to decrease ionic interactions with the stationary phase. However, as has been reported by several authors,[21-25] the analyte ion signal obtained in ESIMS is suppressed by the presence of TFA, even at very low concentrations. Furthermore, the inclusion of TFA or other ion-pairing reagents in the mobile phase raises the conductivity to a level unsuitable for ESI, resulting in spray instability. At a given flow rate, an optimum conductivity range is necessary for producing the most stable spray.[23] This can be accounted for in terms of the electric forces acting on the liquid, which are related to the relaxation time of the liquid. With highly conducting liquids, the relaxation time is too short and can be smaller than the time of formation of a droplet. Mobile phases that have high conductivity can be electrosprayed by the addition of an organic sheath liquid. Another approach has been to use an ultrasonic[21] or pneumatic[25] nebulizer to assist in the formation of a spray. Unfortunately, these methods are not able to eliminate signal suppression caused by TFA. The mechanism of gas-phase ion formation in ESI is still not fully understood[26] nor is the cause of TFA ion signal suppression. Analyte ion signal suppression caused by the presence of TFA is probably a consequence of the weak dependence of the total electrospray current on the total electrolyte concentration. In the charged droplets, the analyte ions compete with other ions among the excess charge on the droplets' surface. A proportionality to concentrations of the ions in the droplets may be expected in this competition. In the case of addition of TFA to the mobile phase, the decrease of peptide ion abundance can be rationalized on the basis of the competition between H_3O^+ ions

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and peptide ions in the conversion process to gas-phase ions. In addition, the formation of strong ion pairs between TFA anions and protonated analyte molecules has been proposed by Apffel et al. to account for signal suppression observed for basic molecules in ESIMS in the presence of TFA.[27] Protonated peptide molecules appear as neutrals in solution, preventing the formation of gas phase ions from charged droplets. Due to the ion pairing, a large portion of the analyte ions is not transferred to the gas phase. Considering the previously discussed problems, TFA must be considered as a mobile-phase additive that is incompatible with ESIMS analysis of peptides at the femtomole level.

To suppress ionization of residual silanols, the pH of the mobile phase can be lowered by the addition of acetic acid instead of TFA. Acetic acid causes less ion suppression than TFA. However, a low pH does not necessarily eliminate silanol effects. Reversed-phase separations of peptides carried out using an aqueous acetic acid/acetonitrile mobile phase often exhibit poor peak shape, nonreproducible retention times, and loss of recovery. To avoid contribution of ionic interactions to retention of peptides, polymer-based columns were employed for peptide separations. The stability of polymeric packings over a wide pH range is an advantage, but in terms of efficiency, silica-based packings are still preferable to their polymer based counterparts.

In recent years, significant progress has been made toward the development of novel silica based stationary phases suitable for the chromatography of basic compounds in simple eluents. It was our aim to select one of the novel silica-based C_{18} packing materials to prepare microcapillary columns. The packing material had to be suitable for the chromatography of MHC class I associated peptides in an ESI-compatible mobile phase. An HPLC fractionated sample generally contains more than 1000 distinct MHC-associated peptides. Obviously, no HPLC column will be able to resolve all peptides completely, so, it is useless to compare the selectivity of the packing materials. Therefore the selection was based solely on the comparison of peak shape.

The performance of the columns was assessed by employing a peptide standard solution containing the hydrophilic peptide angiotensin III (amino acid sequence: RVYIHPF) and the hydrophobic peptide oxytocin (CYISNCPIG-NH_2). Fused silica capillary (100 μm i.d.) was packed with reversed-phase packing material to a length of 20−25 cm. A 500 nL aliquot of the aqueous peptide solution was injected onto the column. The mobile phase was 0.1 M acetic acid in water (A) and 0.1 M acetic acid in acetonitrile (B). Separation was achieved using a linear gradient of 0 to 90% B in 30 min at a flow rate of 0.5 μL/min. Figure 2A shows the separation of the peptides on a Purospher RP-18 column. This column exhibited good peak shapes using an ESI-compatible mobile phase. Of the silica-based C_{18} packing materials evaluated, Purospher RP-18 gave the best peak shapes.

The column length of 25 cm was a compromise between maximizing the resolution and limiting the column back pressure. When a 25-cm column length was used, the back pressure did not exceed 120 bar over the course of the HPLC solvent gradient.

The optimum performance of a 100 μm-i.d. column is attained at a flow rate of ~0.5 μL/min, while optimal ESI performance is obtained at flow rates of 1−3 μL/min. The flow rate generated

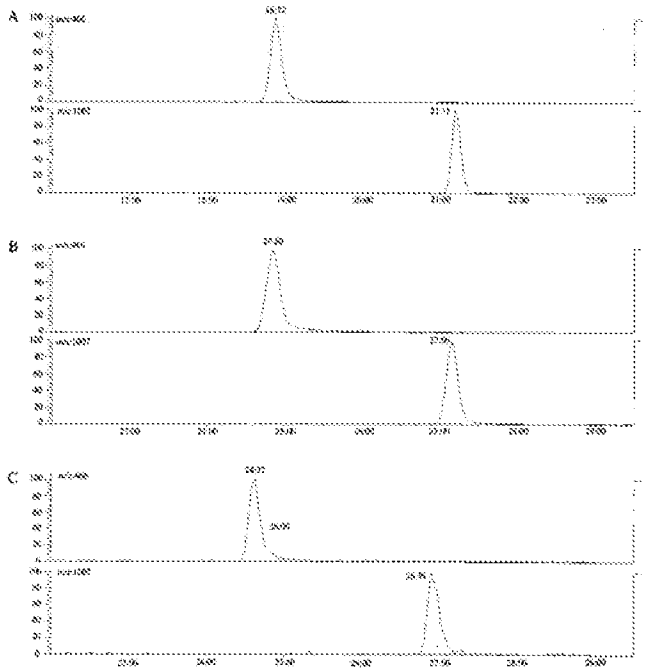

Figure 2. Microcapillary HPLC−ESIMS extracted ion chromatograms of 2.5 pmol (A), 5 pmol (B), and 100 fmol (C) each of angiotensin III and oxytocin. Data were acquired by scanning the mass spectrometer from *m/z* 400 to 1050. Angiotensin III ($[M + 2H]^{2+}$ ion, *m/z* 466), oxytocin ($[M + H]^+$ ion, *m/z* 1007). (A) Peptides were separated on a 250 mm × 100 μm Purospher RP-18 column. Injection volume, 500 nL. (B, C) Peptides were loaded and preconcentrated on a 7 mm × 100 μm Alltima C_8 column and separated on a 250 mm × 100 μm Purospher RP-18 column. Injection volume, 30 μL.

by microcapillary HPLC can easily be augmented by the addition of a makeup solvent. Although the addition of a makeup solvent results in dilution of the analytes eluting from the column, the presence of methanol in a makeup solvent lowers both the surface tension and the conductivity of the column effluent, thereby enhancing the signal obtained from the peptides. This increase in signal more than compensates for the decrease in signal caused by dilution. In terms of sensitivity, the best results were obtained by using a solvent of 70% methanol and 30% water in a ratio of 3:1 (v/v) to the column flow. Usually the makeup solvent is introduced coaxially by means of a sheath liquid tube and mixed with the HPLC effluent at the tip of the electrospray needle. However, the transfer of the HPLC effluent to the tip of the electrospray needle through a fused-silica capillary of 30-cm length, the minimum length dictated by the dimensions of the electrospray probe, at a flow rate of 0.5 μL/min caused a significant extracolumn band broadening. The addition of a makeup solvent by means of a microvolume tee at the outlet of the analytical column (Figure 1) increases the flow rate through the transfer capillary to 2 μL/min and reduces band broadening. Microcapillary columns with inner diameters of 50−75 μm have been used for the separation of peptides.[8,18,28] Compared to 100 μm-i.d. columns, these smaller diameter columns offer the advantage of decreased elution volume and, hence, increased concentration of

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the analyte. However, because of the unavoidable addition of makeup solution to the column effluent, the concentration of the analytes at the electrospray needle tip does not increase by applying 50- or 75-μm i.d. columns rather than 100-μm i.d. columns. Considering the more stringent demands put on the extracolumn volume by 50- and 75-μm i.d. columns, we decided to employ 100-μm i.d. columns.

Selection of the Precolumn. The first attempt to develop a microcapillary column switching system that performed properly involved a combination of a 300-μm i.d. precolumn and a 100-μm i.d. analytical column. Compared to a 100-μm i.d. precolumn, a 300-μm i.d. precolumn has the advantage of a larger sample load capacity. In addition, by using a short length of packing material, a flow rate of 100 μL/min can be easily maintained at a reasonable column back pressure, allowing fast injection of 30−50-μL sample volumes. Unfortunately, separation of peptide standards using a 7 mm × 300 μm precolumn packed with Alltima C_8 and a 25 cm × 100 μm analytical column packed with Purospher RP-18 resulted in severe peak broadening (data not shown). Peak widths up to 4 min were observed. Substitution of a Purospher RP-18 precolumn for the Alltima C_8 precolumn did not improve peak shapes. To clarify these results, it is important to note that, after switching the six-port valve (Figure 1), the flow rate through the precolumn was reduced from 100 to 0.5 μL/min. The optimal solvent flow rate for a 300-μm i.d. column is 4−5 μL/min. Hence peptides elute from the precolumn at a flow rate far below the optimum; this is probably the cause of peak tailing. High separation efficiencies using microcolumn switching systems consisting of a 500−800-μm i.d. precolumn and a 300-μm i.d. analytical column have been demonstrated.[29−31] To avoid migration of analytes through the precolumn, these systems applied backflushing of the retained analytes from the precolumn onto the analytical column at a flow rate of 3−5 μL/min. However, backflushing the precolumn requires the construction of a second frit in the precolumn. It is rather difficult to construct a second frit in the precolumn without causing extracolumn band broadening when a 100-μm i.d. analytical column is used.

The aforementioned problems led us to the decision to employ 100-μm i.d. precolumns. The ion chromatograms displayed in Figure 2B were obtained using a 7 mm × 100 μm Alltima C_8 precolumn. A comparison of Figure 2A and B reveals that some band broadening still occurs, but in our opinion the shape and width of the peaks are acceptable. Increasing the injection volume to 50 μL did not have an adverse effect on the peak width.

With respect to peak shape, a 7 mm × 100 μm Purospher RP-18 precolumn performed equally well. However, separation of peptide standards using the Purospher RP-18 precolumn revealed partial breakthrough of angiotensin III even when only 500 fmol was injected onto the precolumn. The Alltima C_8 precolumn did not show any breakthrough of angiotensin III. Increasing the length of the Purospher RP-18 precolumn to 20 mm was necessary to eliminate sample breakthrough. Breakthrough of angiotensin III indicates that this peptide migrates through the precolumn even with a mobile phase of 0.1 M acetic acid in H_2O. Therefore, Purospher RP-18 is not suitable as packing material for reliable trapping of peptides and Alltima C_8 was selected for the preparation of precolumns.

The sample load capacity of a 7 mm × 100 μm Alltima C_8 precolumn was determined by injecting 30 μL of the peptide standard solution containing different amounts of angiotensin III and oxytocin. It was found that at least 10 pmol (total amount of peptide) could be injected onto the precolumn without causing breakthrough. The sample load capacity can be increased by increasing the length of packing material. However, peak width was observed to increase with precolumn length. Therefore, all further experiments were performed with a 7 mm × 100 μm Alltima C_8 precolumn. The short length of packing material allows a flow rate of 10 μL/min through the precolumn during sample loading at a reasonable low column back pressure of ~50 bar.

Besides by insufficient trapping, loss of peptides can occur as a result of irreversible adsorption on the precolumn. The recovery of the peptides from the precolumn was estimated by comparing the peak areas of the two peptide standards obtained by performing the separation using the column switching system (30-μL injection) with the peak areas obtained by carrying out the separation using only the analytical column (500-nL injection). The recovery was determined at a concentration level of 167 and 17 fmol/μL (5 pmol and 500 fmol injected onto the precolumn, respectively). The average recovery of angiotensin III and oxytocin was 90−100%. The sensitivity of the system is illustrated in Figure 2C, which shows the separation of 100 fmol each of angiotensin III and oxytocin. Figure 3 demonstrates the separation of six synthetic, 9-residue peptides.

It must be emphasized that the microcapillary column switching system is not suitable for the analysis of small, hydrophilic peptides. Analyses of tryptic digests of several proteins showed a loss of small, hydrophilic peptides of 3−6 residues because of insufficient retention of these species on the precolumn. This poses no problem for the analysis of MHC class I associated peptides since these peptides vary in length from 8 to 10 amino acids.

Identification of MHC Class I-Associated Peptides. Peptides associated with HLA-A*0201 molecules were extracted from both uninfected cells and measles virus infected cells. After HPLC fractionation, aliquots of each fraction were analyzed by microcapillary HPLC−ESIMS. At every stage of the procedure, samples from uninfected cells and virus infected cells were treated identically. Particular attention was paid to the reproducibility of the HPLC fractionation. The retention time reproducibility was checked at regular intervals using a mixture of 9-residue, synthetic peptides. A comparison of the results obtained from the analysis of HPLC fractions of measles virus infected cells with the results obtained from the analysis of the corresponding HPLC fractions of uninfected cells should provide the molecular mass of peptides arising as a result of measles virus infection, i.e., peptides unique to infected cells. These peptides would be expected to derive from viral proteins but might also derive from endogenous proteins. Figure 4 shows the reconstructed ion current (RIC) chromatograms from the HPLC−ESIMS analysis of peptides eluted from HLA-A*0201 molecules purified from either infected or uninfected cells. Obviously, because of the overwhelming complexity of the

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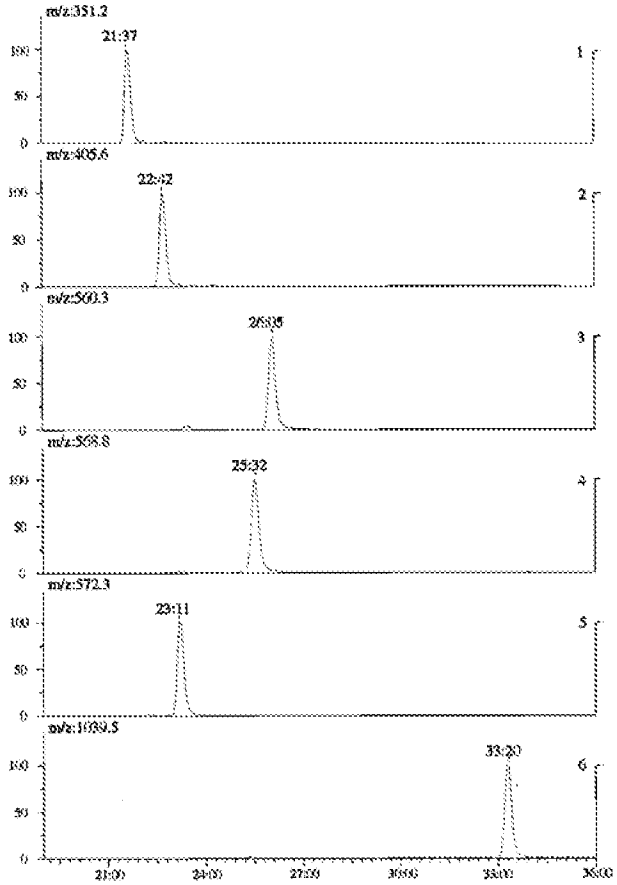

Figure 3. Microcapillary HPLC−ESIMS extracted ion chromatograms of 2.5 pmol each of 9-residue synthetic peptides. Peptides were loaded and preconcentrated on a 7 mm × 100 μm Alltima C_8 column and separated on a 250 mm × 100 μm Purospher RP-18 column. A 30-μL aliquot of the peptide solution was injected onto the precolumn. Data were acquired by scanning the mass spectrometer from *m/z* 300 to 1250: (1) RMSKGVFKV ($[M + 3H]^{3+}$, *m/z* 351.2, 21:37); (2) RLERKWLDV ($[M + 3H]^{3+}$, *m/z* 405.6, 22:42); (3) LMIDRPYVL ($[M + 2H]^{2+}$, *m/z* 560.3, 26:05); (4) DLSLRRFMV ($[M + 2H]^{2+}$, *m/z* 568.8, 25:32); (5) HLMIDRPYV ($[M + 2H]^{2+}$, *m/z* 572.3, 23:11); (6) LLWSYAMGV ($[M + H]^{+}$, *m/z* 1039.5, 33:20)

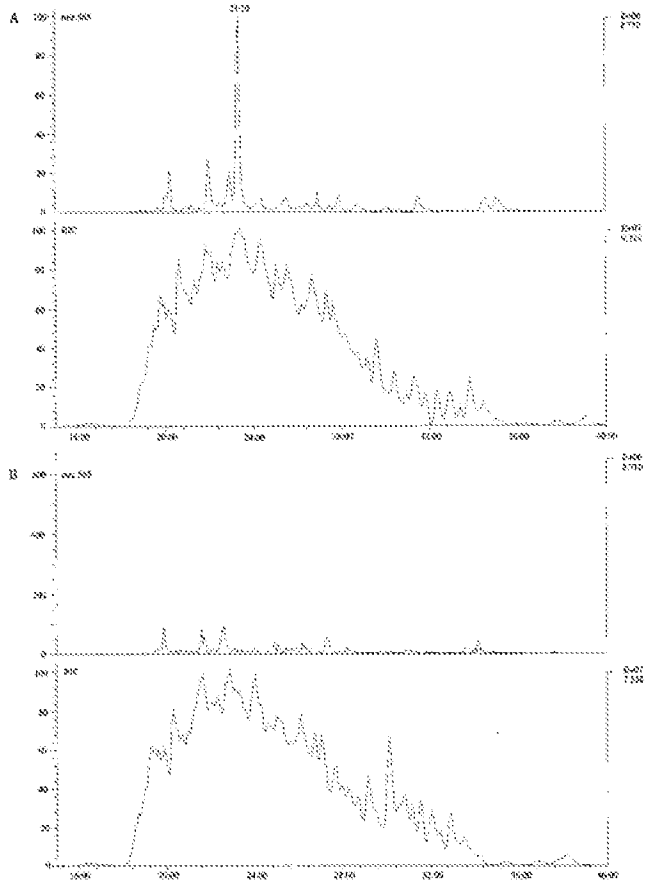

Figure 4. Microcapillary HPLC−ESIMS ion chromatograms of peptides extracted from 2.75 × 10^6 cells. The ion chromatograms for *m/z* 565 ± 0.5 and the RIC chromatograms were obtained from the analysis of a 30-μL aliquot of HPLC fraction 2 of measles virus infected cells (A) and uninfected (B) cells. The mass spectrometer was scanned from *m/z* 400 to 1500 at a resolution of 1500. Approximately 30% of the HPLC fraction was consumed to obtain the data.

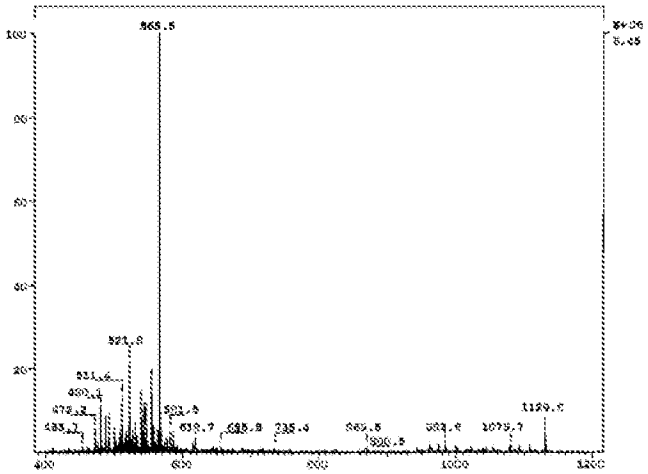

Figure 5. ESI mass spectrum obtained from the chromatographic peak centered at 23:19 retention time shown in Figure 4A. The spectrum shows the doubly charged molecular ion ($[M + 2H]^{2+}$) at *m/z* 565.5 and singly charged molecular ion ($[M + H]^{+}$) at *m/z* 1129.6 of a peptide unique to measles virus infected cells.

samples, the RIC chromatograms are virtually identical and are not suitable for the detection of virus specific peptides. On the other hand, the ion chromatogram for *m/z* 565 obtained from the analysis of virus infected cells (Figure 4A) clearly shows a peak at a retention time of 23:19 min, which is not observed in the *m/z* 565 ion chromatogram of uninfected cells (Figure 4B). The ESI mass spectrum corresponding to the peak at 23:19 min retention time (Figure 5) exhibits a doubly charged ion at *m/z* 565.5 and a singly charged ion at *m/z* 1129.6, thus revealing an average monoisotopic molecular mass of 1128.8 Da for this peptide (herein referred to as peptide 1128).

The ion chromatograms of each HPLC fraction from virus infected cells were compared to the ion chromatograms of the corresponding HPLC fraction from uninfected cells over the scan range of the mass spectrometer, using a mass window of 1 Da for each ion chromatogram. This approach is laborious and time-consuming but will eventually reveal the molecular mass of most, if not all, naturally processed peptides unique to measles virus infected cells that are present in quantities exceeding the limit of detection of the method and are capable of binding to MHC class I molecules. The presence of low-abundance peptides in extracts from both uninfected and virally infected cells produced background signals that established the threshold of detection at 5−25

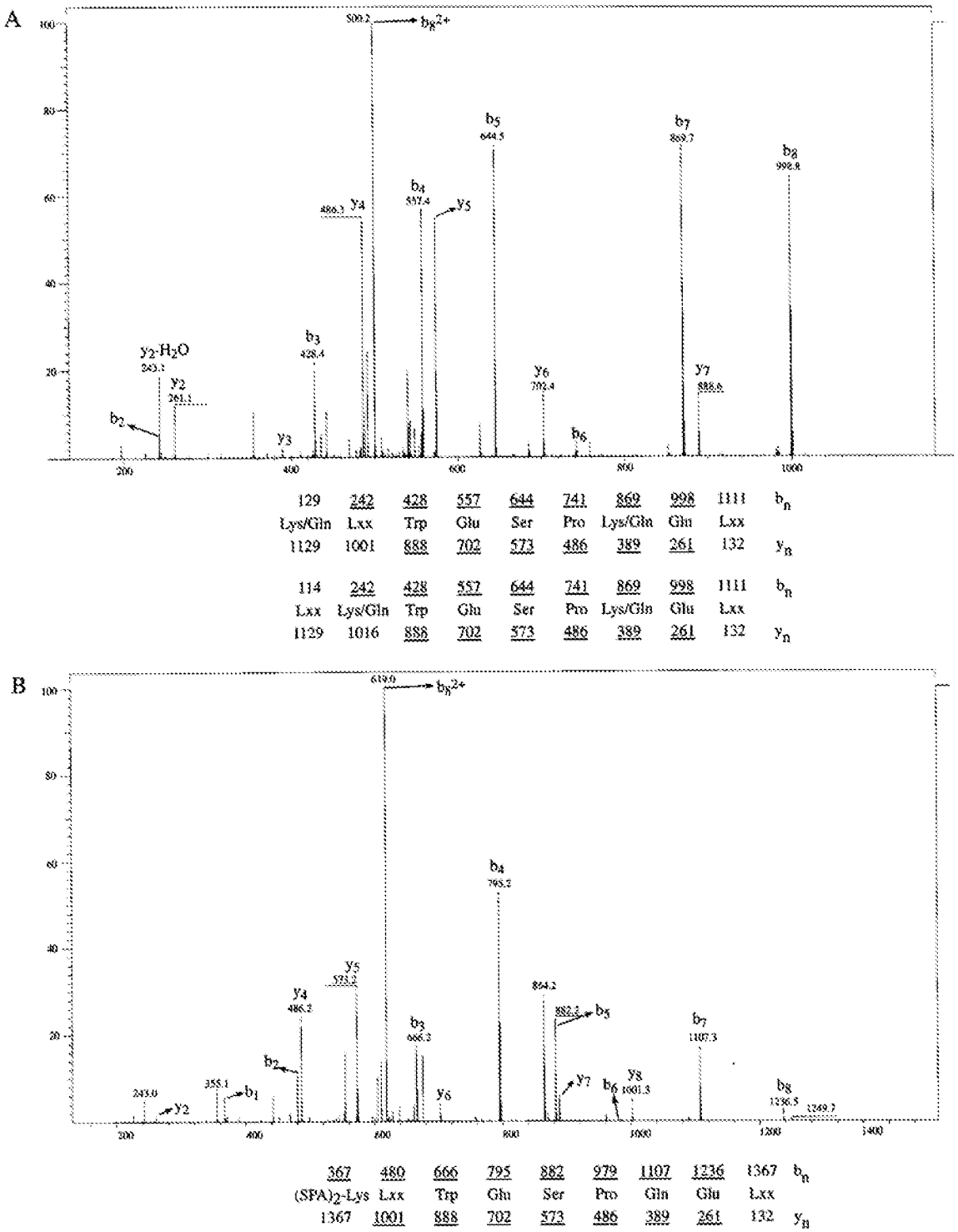

Figure 6. CID mass spectra obtained from the microcapillary HPLC–ESIMS/MS analysis of a 30-μL aliquot of HPLC fraction 2 of measles virus infected cells. (A) CID mass spectrum of the $[M + 2H]^{2+}$ ions with *m/z* 565.5 of a candidate antigenic peptide with a molecular mass of 1128.8 Da. (B) CID mass spectrum of the $[M + 2H]^{2+}$ ions with *m/z* 684.5 of the SPA-derivatized candidate antigenic peptide. The molecular mass of the peptide after derivatization was 1366.8 Da. The mass spectrometer was scanned from *m/z* 155 to 1150 (A) or from *m/z* 190 to 1850 (B). Note that the lower limit of the scan range is determined by the minimum storage *m/z* of the ion trap, which is dependent on the *m/z* of the precursor ion. Predicted nominal masses for fragment ions of types b and y are shown above and below the deduced amino acid sequence, respectively. Ions observed in the spectrum are underlined. Lxx represents leucine or isoleucine.

fmol (amount injected onto the column). Peptides present in both samples were used as markers to correct for retention time shifts. The principal limitation of this approach arises from the presence of dominant peptides derived from cellular proteins. Coelution of these self peptides with low-abundance viral peptides of identical molecular mass may obscure the ion signals of the latter species, preventing their detection.

To sequence the naturally processed peptide 1128, the microcapillary column-switching HPLC system was coupled to an ion trap mass spectrometer. The doubly charged molecular ion was

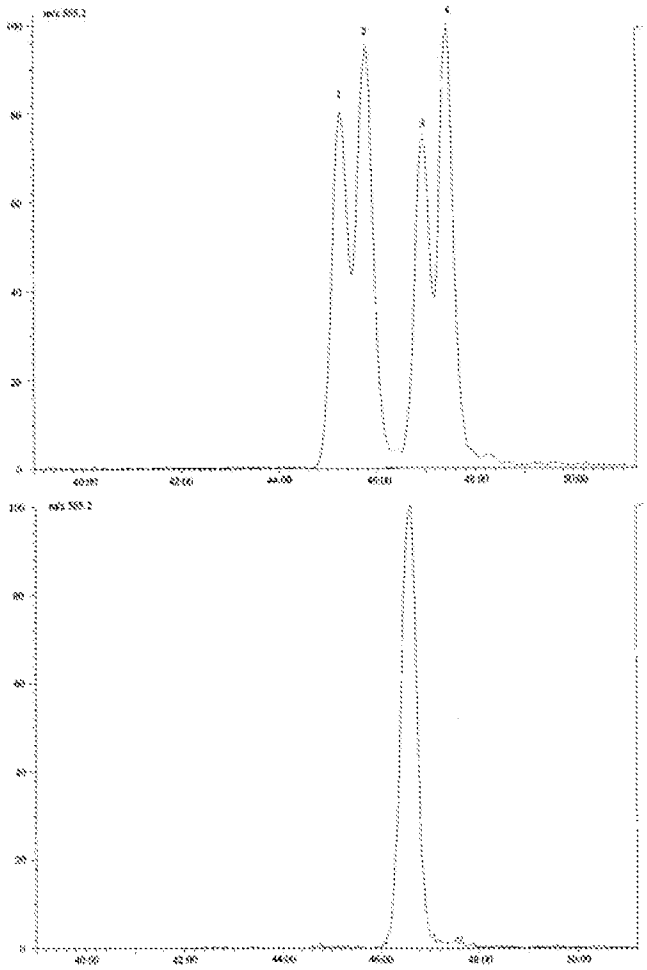

Figure 7. Coelution of KLWESPQEI with the naturally processed peptide. Peptides were loaded and preconcentrated on a 7 mm × 100 μm Alltima C_8 column and separated on a 250 mm × 100 μm Purospher RP-18 column. The mobile phase used for gradient elution consisted of (A) 0.1 M acetic acid in water and (B) acetonitrile containing 0.1 M acetic acid. Peptides were separated using a gradient of 0.5% acetonitrile/min. Data were acquired by scanning the mass spectrometer from *m/z* 500 to 1200. Shown are the extracted ion chromatograms of *m/z* 565 ($[M + 2H]^{2+}$ ion). (A) Standard solution of synthetic peptides (1 pmol each): (1) KIWESPQEI, (2) KIWESPQEL, (3) KLWESPQEI, and (4) KLWESPQEL. (B)HPLC fraction 2 of measles virus infected cells to which synthetic KLWESPQEI had been added.

selected to undergo CID, yielding the mass spectrum shown in Figure 6A. The amino acid sequence deduced from the MS/MS spectrum is either ZXWESPZEX or XZWESPZEX. The letter X is used to designate either leucine or isoleucine and Z represents lysine or glutamine, which cannot be distinguished by low-resolution MS. Based on the MS/MS spectrum it was not possible to determine the order of the first two amino acids, because the *m/z* of the b_1 ion was below the minimum storage *m/z* of the ion trap and the y_8 ion was not observed in the spectrum. However, the observation of the b_2 ion at *m/z* 242 only allows the combination XZ or ZX for the first two residues in the sequence. The corresponding HPLC fraction of uninfected cells was also analyzed by microcapillary HPLC–ESIMS/MS. The results confirmed that peptide 1128 was not present in this sample (data not shown).

The next step in the identification procedure involved derivatization of peptides with SPA. The HPLC fraction containing peptide 1128 was subjected to on-column SPA derivatization. SPA reacts with the N-terminal and the lysine amino groups of the peptides, forming *N*-pyridylacetyl derivatives.[18,20] Thus, the SPA derivatization enables the differentiation of lysine from glutamine. In addition, considering the sequence of peptide 1128, the derivatization will add 119 Da to the N-terminal amino acid if the peptide has leucine, isoleucine, or glutamine at this position or 238 Da if the residue at position 1 is lysine. In both cases, the *m/z* of the b_1 ion is raised above the minimum storage *m/z* of the ion trap, allowing the identification of the amino acid residue at position 1. LC–MS analysis of the HPLC fraction after SPA derivatization revealed a retention time shift of ~10 min for the peptide (data not shown). The *m/z* shift observed for the $[M + 2H]^{2+}$ and $[M + H]^{+}$ ions upon derivatization was 119 and 238, respectively, indicating that the peptide contains a single lysine residue. The MS/MS spectrum of the derivatized peptide (Figure 6B), obtained by CID of the $[M + 2H]^{2+}$ ion at *m/z* 684.5, shows a complete series of b ions. The b_1 ion at *m/z* 367 establishes a definitive assignment of lysine at position 1. The sequence obtained from the MS/MS spectrum is KXWESPQEX.

Examination of the known amino acid sequence of measles virus proteins revealed that only one of the four remaining candidate peptides could originate from a viral protein, KLWESPQEI. The source protein for this peptide is the measles virus nonstructural protein C (residues 84–92). Based on the knowledge of peptide binding motifs, a high affinity of KLWESPQEI for HLA-A*0201 is predicted.

To identify the naturally processed peptide, four peptides were synthesized with I or L at positions 2 and 9 of KXWESPQEX. Using microcapillary HPLC with a shallow gradient enabled the partial separation of these synthetic peptides (Figure 7A). Addition of synthetic KLWESPQEI to the sample revealed a coelution of KLWESPQEI with the naturally processed peptide (Figure 7B). On the basis of the observation that only KLWESPQEI, and none of the other isoforms, coeluted with the naturally processed peptide, we concluded that KLWESPQEI is the viral peptide bound to HLA-A*0201 molecules. The CID mass spectrum of the naturally processed peptide closely matched that of synthetic KLWESPQEI (data not shown), both with respect to the appearance of specific daughter ions and to their relative abundance.

The known quantity of synthetic KLWESPQEI added to the cell extract allowed the quantification of naturally processed KLWESPQEI. The total amount of naturally processed peptide in the HPLC fraction, which was calculated from the signal increase upon addition of the synthetic peptide, appeared to be ~80 pmol. This amount was extracted from 2.75×10^9 cells, indicating that this peptide is present on the surface at 17 000 copies/cell. Losses during peptide extraction and HPLC fractionation were not taken into account. Peptide binding assays and immunogenicity studies were conducted; the results will be published in a separate paper.[32]

Approximately 50 peptides were detected that appeared only in the samples derived from virally infected cells, with

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KLWESPQEI representing the predominant species. The sequencing of these peptides is in progress and the results of this work will be published in a separate paper.

CONCLUSIONS

A microcapillary column switching HPLC–ESIMS system has been developed for the identification of peptides presented by MHC class I molecules. Peptides unique to virus infected cells are detected and identified by the comparison of LC–MS data obtained from the analysis of extracts from virally infected cells and their uninfected counterparts. The suitability of this approach was demonstrated with the identification of a naturally processed peptide derived from a measles virus protein. A limitation of the method arises from the presence of dominant self-peptides. Coelution of these peptides with low-abundance viral peptides of identical nominal molecular mass may prevent the detection of the latter species. We are currently studying the significance of this limitation. The identification of peptides arising as a result of measles virus infection is in progress. We found that usually only a partial amino acid sequence can be obtained from ion trap tandem MS spectra. Additional sequence information can often be obtained by the application of on-column derivatization.

A problem that has to be addressed is the unknown loss of peptides during extraction and purification. Although the overall efficiency of peptide recovery has been estimated,[11,33] an accurate quantification of identified T-cell epitopes requires the determination of the yield for each distinct peptide.

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